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A cultivable acoel species from the Mediterranean, *Aphanostoma pisae* sp. nov. (Acoela, Acoelomorpha)

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Abstract

Aphanostoma pisae **sp. nov.** is an interstitial acoel found at the coast of the Liguric Sea in Pisa (Tuscany, Italy). It belongs to the large family Isodiametridae, characterised by a male copulatory organ with a cylindrical shape and non-anastomising longitudinal muscle fibers. It is the first recognised species of *Aphanostoma* in the Mediterranean and it can occur in great abundance at its type locality (several hundred specimens in a spoonful of sand). *A. pisae* has been cultured in the laboratory for several years with diatoms for food. The embryonic development lasts for just under two days at 20 °C.

We provide a description of the new species using live observations, light and electron microscopy of sagittal sections and stainings of the filamentous actin and the serotonergic nervous system, and we discuss and update the genus diagnoses of the genera *Aphanostoma* and *Praeconvoluta*.

Key words: Xenacoelomorpha, acoel flatworm, taxonomy, phalloidin, antibody stainings

Introduction

Acoels are predominantly marine worms with a contested phylogenetic position. For more than a century, acoels were considered members of the Platyhelminthes based on many morphological similarities (Ehlers, 1985), but a great number of molecular analyses now place them either as sister group of the Bilateria (Ruiz-Trillo *et al.*, 1999, Egger *et al.*, 2009) or, most recently, as deuterostomes (Philippe *et al.*, 2011). Identification of acoels requires careful observation and in many cases sagittal sections of the genital organs of mature animals are required for identification (Westblad, 1948). Staining of the musculature (F-actin) can provide an additional level of detail for characterising these organs and the body wall musculature (Hooge, 2001; Hooge & Tyler, 2005).

While many Scandinavian and Northern German acoel species have been described, the Mediterranean (and even more so most other parts of the world) are still comparatively poorly covered (Nilsson *et al.*, 2011). This trend is reflected in the absence of recognised species of the genus *Aphanostoma* Ørsted, 1845 in the Mediterranean. With this work, we provide the first description of an *Aphanostoma* species in the Mediterranean with images of live animals, sagittal sections, and stainings of the musculature and the serotonergic nervous system. We also propose a revision of the closely related genera *Aphanostoma* and *Praeconvoluta* Dörjes, 1968.

Material and methods

Sampling. Sand samples from Marina di Pisa, Italy (43.6761°N 10.2698° E) were taken in May 2005 and May 2011. Animals were extracted from the sand in 7% $MgCl_2 \cdot 6H_2O$ mixed 1:1 with artficial sea water and then transferred to petri dishes (see below).

Cultures. *Aphanostoma pisae* was cultured in petri dishes with enriched sea water (f/2 medium) and fed *ad libitum* with the diatom *Nitzschia curvilineata* in a constant environment at 20°C with a day/night cycle of 12/12 hours. About every two weeks, algae were replaced if necessary. When starting new dishes, about 40 adult worms

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were put into a new, clean dish and algal f/2 suspension was added until the dish was half-filled. For the preparation of algae see Egger & Ishida (2005).

Semi- and ultra-thin sagittal serial sections. Mature animals were relaxed in 7% $MgCl_2 \cdot 6H_2O$, and fixed using three different protocols. 1) fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) containing 10% sucrose for 1 hour on ice, and then post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.3) for 45 min, washed in cacodylate buffer for 15 min and in distilled water for 5 min, dehydrated in a standard acetone series and embedded in Spurr's resin (Spurr, 1969) or Mollenhauer (Mollenhauer, 1964). 2) fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS) containing 10% sucrose for 1 hour, washed in distilled water for 5 min and dehydrated in a standard ethanol series and embedded in Spurr's resin. 3) fixed for electron microscopy following a slightly modified protocol from Eisenman & Alfert (1982) for an acoel species (Salvenmoser *et al.* 2010).

Semi-thin sections of 5 animals were made at 1–2 µm thickness using an Autocut microtome (Reichert, Vienna, Austria) and a histo Butler diamond knife (Diatome, Biel, Switzerland) and were mounted on slides. Sections were stained according to Richardson *et al.* (1960) or to Heidenhain (Romeis, 1968; Nimeth *et al.* 2007). Ultra-thin sections of 1 specimen were made at 80 nm thickness using an ultramicrotome UCT (Leica, Vienna, Austria), stained with lead citrate and mounted on filmed 50-mesh, and 200-mesh thinbar grids.

Phalloidin and serotonin stainings. Mature animals were relaxed in 7% $MgCl_2 6H_2O$, fixed in 4% formaldehyde (from paraformaldehyde) in PBS for 1 hour, washed in PBS-T (PBS with 0.1% Triton-X) for 30 min and blocked in BSA-T (PBS-T with 1% bovine serum albumine) for 1 hour. For phalloidin stainings, animals were incubated in rhodamine-conjugated phalloidin (Sigma-Aldrich, St. Louis, MO) 1:600 in BSA-T for 1 hour in the dark, while for stainings of the serotonergic nervous system the primary antibody was applied (rabbit-anti-serotonin, Sigma-Aldrich) 1:1500 in BSA-T overnight in the fridge, which was followed by washes in PBS-T for 30 min, blocking in BSA-T for 30 min and incubation with the secondary antibody (swine-anti-rabbit, FITC-conjugated, Dako, Glostrup, Denmark) 1:300 in BSA-T for 1 hour in the dark. After that, for both the phalloidin and the anti-serotonin stainings, animals were washed in PBS-T for 30 min and mounted in Vectashield (Vector Labs, Burlingame, CA).

Microscopy. Live animals in squeeze preparations and sagittal sections were observed with a Leica DM5000B, a Zeiss Axio Imager M.1 light microscope, and a Zeiss Libra 120 EFTEM electron microscope. Pictures were made using Penguin Pixera 600CL, Leica DFC490, Zeiss AxioCamHR and TRS 2048 high speed digital cameras. Confocal stacks were made using a Zeiss 510 laser scanning microscope. Figures were made using GIMP (http://www.gimp.org) and ImageJ (http://imagej.nih.gov/ij), and schemes were drawn with Inkscape (http://www.inkscape.org).

Type material. Holotype and paratype were deposited at the Natural History Museum Vienna, Austria (NHMW).

Results

Systematics

Family Isodiametridae Hooge & Tyler, 2005

Genus *Aphanostoma* Ørsted, 1845

Aphanostoma pisae sp. nov.

Type material. Holotype (NHMW ZOOEV MIKRO 5597/1-2): A complete set of serial sagittal 1-µm sections of a cultured mature adult from the Pisa excursion in 2005 on 2 microscopic slides, Heidenhain staining, coverslipped in cedar wood oil.

Paratype (NHMW ZOOEV MIKRO 5598/1-3): A complete set of serial sagittal 1-µm sections of a cultured mature adult from the Pisa excursion in 2011 on 3 microscopic slides, Heidenhain staining, coverslipped in cedar wood oil.

Type locality. Marina di Pisa, Italy (43.6761°N 10.2698° E). Intertidal wet sand taken from surface and ca. 20 cm deep. Beach protected by an artificial embankment of rocks.

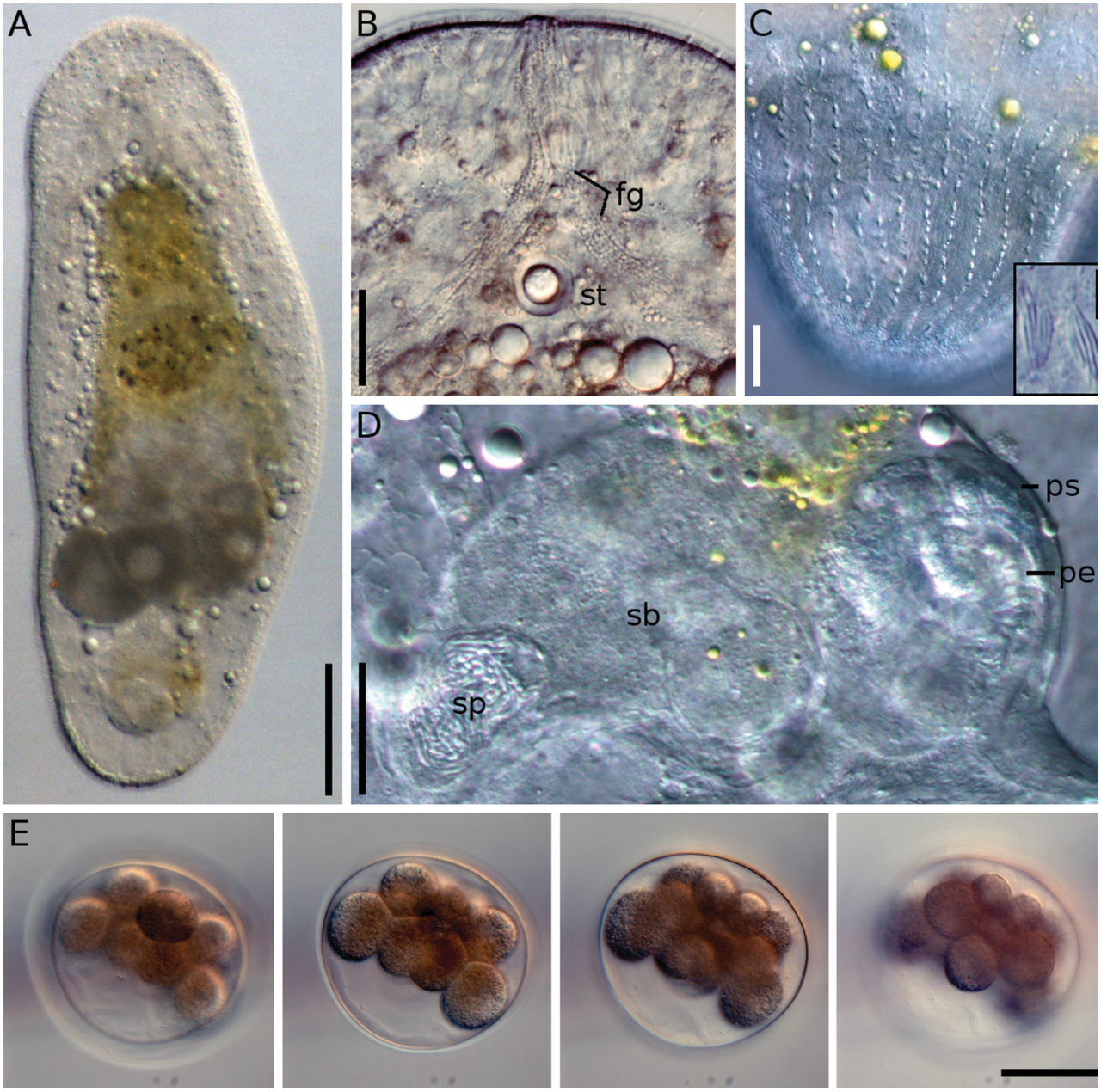

FIGURE 1. Live differential interference contrast images of adult animals (A–D) and of an embryo (E). (A–C) Anterior to the top. (A) Mature specimen. (B) Detail of the anterior tip with frontal glands *fg* and the statocyst *st*. (C) Rhabdoid glands in rows at the posterior end; inset shows detail of rhabdoid glands. (D) Genital organs, anterior to the left. Note foreign sperm *sp* at anterior part of the seminal bursa *sb*. (E) Focus series through a 12-cell stage embryo. *pe* penis, *ps* penis sheath. Scale bars: 100 µm in (A), 25 µm in (B–D), 5 µm in (C) inset, 50 µm in (E).

Etymology. Specific epithet named after the type locality, (Marina di) Pisa in Italy.

Morphology. Body elongate to drop-shaped, posterior end narrower than anterior end (Fig. 1A). Transparent, mature specimens 400–700 µm long (500 µm ± 97 µm, n=7) and 160–280 µm wide (217 µm ± 42 µm, n=7) at their widest point.

Epidermis multiciliated. Rhabdoid glands arranged in longitudinal rows (Fig. 1C). Glands of the frontal organ reach from the anterior-most tip of the animal and split left and right anterior of the statocyst, each side extending posteriorly to the digestive syncytium (Fig. 1B). Eyes or pigment spots absent. Statocyst in anterior fifth of the body (Figs. 1AB). Mouth ventral and central (Figs. 2A, 3A), without pharynx. Large chordoid vacuole located at the posterior tip (Figs. 1A, 2D, 4B), many smaller chordoid vacuoles on the dorsal and less on the ventral side up to and posterior of the statocyst.

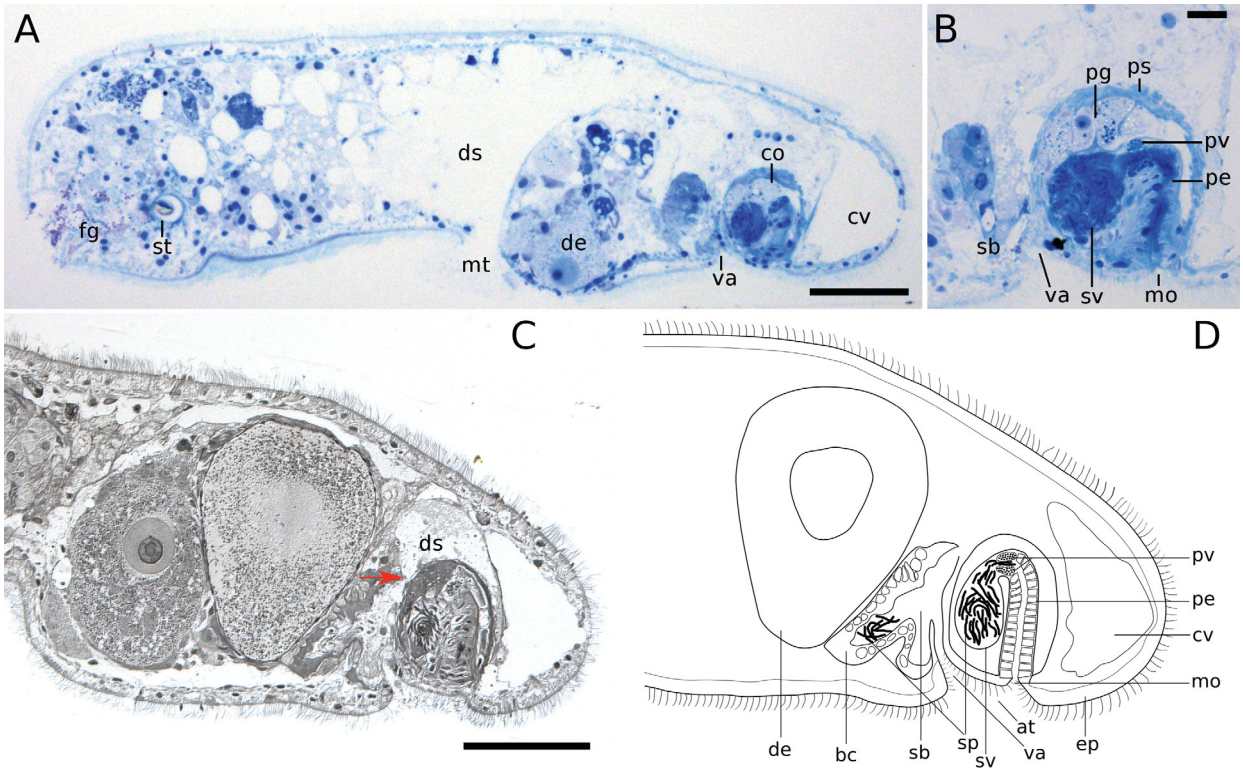

FIGURE 2. Sagittal sections of mature animals, anterior to the left, dorsal up. (A–B) Stained after Richardson *et al.* (1960). (C) Stained after Heidenhain (Romeis, 1968). Red arrow marks the opening of the bursa to the digestive syncytium. (D) Schematic drawing, based on sections of several individuals. *at* atrium, *bc* bursal cap, *co* copulatory apparatus, *cv* chordoid vacuole, *de* developing egg, *ds* digestive syncytium, *ep* ciliated epidermis, *fg* frontal glands, *mo* male genital opening, *mt* mouth, *pe* penis, *pg* prostatic glands, *ps* penis sheath, *pv* prostatic vesicle, *sb* seminal bursa, *sp* sperm, *st* statocyst, *sv* seminal vesicle, *va* vagina. Scale bars: 50 µm in (A, C), 10 µm in (B).

Testes dorsal, ovaries ventral. Both gonads paired and extending from about the anterior tip of the digestive syncytium to the seminal bursa. Gonopores ventral and separate, but close together, opening into a long, shallow common genital atrium (Figs. 2, 4B). Female genital opening with unciliated vagina lined with prominent microvilli (Fig. 4H) anterior to male genital opening. Weak vaginal sphincter musculature present, but otherwise vaginal musculature is absent (Fig. 3ACD). Seminal bursa without bursa nozzle. Ventral part of bursa is split into anterior (bursa cap) and posterior cove (Figs. 2CD, 4AB), while dorsal part of seminal bursa opens into digestive syncytium as a ductus genito-intestinalis (Figs. 2CD, 4C), where musculature can be found even inside the bursa wall (Fig. 4C). Seminal bursa surrounded by circular musculature as part of the bursa wall, except posteriorly (Figs. 3ACD, 4). Bursa wall electron-dense, non-sclerotised and non-epithelial (Figs. 2CD, 4), thickly lamellated especially dorsally and at the ventral bursa cap (Fig. 4CG,H). Non-sclerotised penis isodiametric, approximately 35 µm in length, ending bluntly at the distal end (Figs. 2, 4B). Penis with outer circular and inner longitudinal muscle fibers which are non-anastomising (Fig. 3CD). Prostatic vesicle at proximal end of the penis, with prostatic glands reaching into the penis (Fig. 2BD). Seminal vesicle directly attached the inverted penis and with its own musculature (Fig. 3C, sv) independent of the thick muscle fibers of the penis sheath (Fig. 3C, ps) and its own epithelium (Fig. 4D–F). Male antrum and false seminal vesicle absent.

Body wall musculature consists of outer circular and inner longitudinal muscle fibers (Fig. 3AB). Straight longitudinal fibers reach from anterior to posterior on the dorsal side (Fig. 3B). Diagonal fibers on the dorsal and especially on the ventral side often start as longitudinal fibers that bend towards the median (Fig. 3AB). Very thick wedge-shaped parenchymal fibers dominate the ventral/midbody side, starting a short distance behind the anterior tip; more posteriorly and reaching almost to the level of the mouth, thick irregularly transversal fibers are apparent on the ventral side (Fig. 3A). The mouth is surrounded by circular musculature, and on both sides of the mouth characteristic longitudinal fibers stretch from anterior and join in a U-shape posterior of the mouth. Ventral thin longitudinal fibers stretch from the posterior side of the mouth towards the bursa (Fig. 3A). Dorsoventral musculature goes straight or in sigmoid curves (Fig. 3CD).

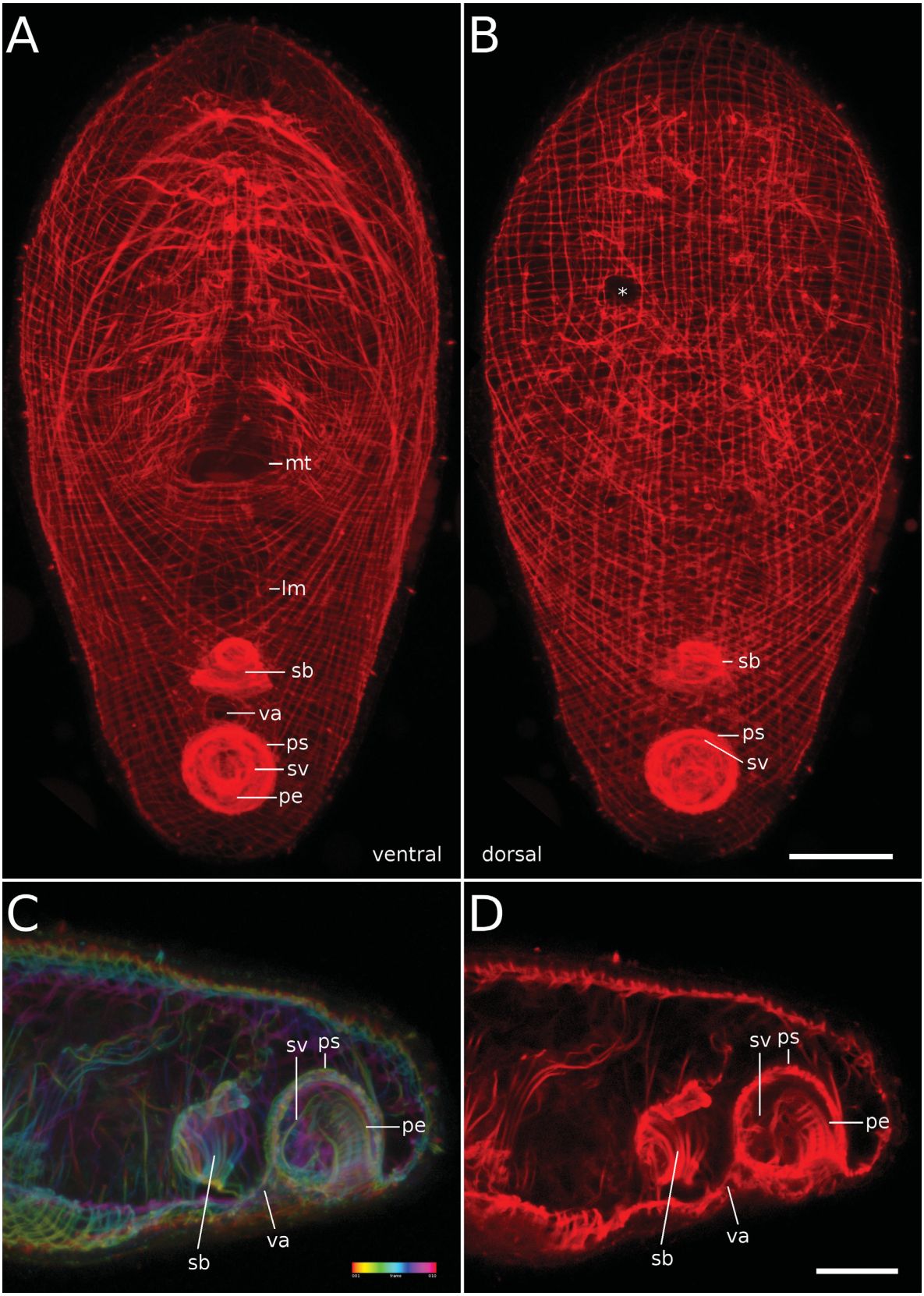

FIGURE 3. Filamentous actin stainings of mature animals. Ventral (A) and dorsal (B) projection of a confocal stack, anterior to the top. (C–D) Anterior to the left. (C) Depth-coded lateral confocal stack of the posterior end, depth values from left to right in coloured scale. (D) Single image of a lateral confocal stack of the posterior end. *lm* longitudinal muscle fibers, *mt* mouth, *pe* penis, *ps* penis sheath, *sb* seminal bursa, *sv* seminal vesicle, *va* vagina. Asterisk marks an artefactual hole in the muscle pattern. Scale bars: 50 µm in (A–B), 25 µm in (C–D).

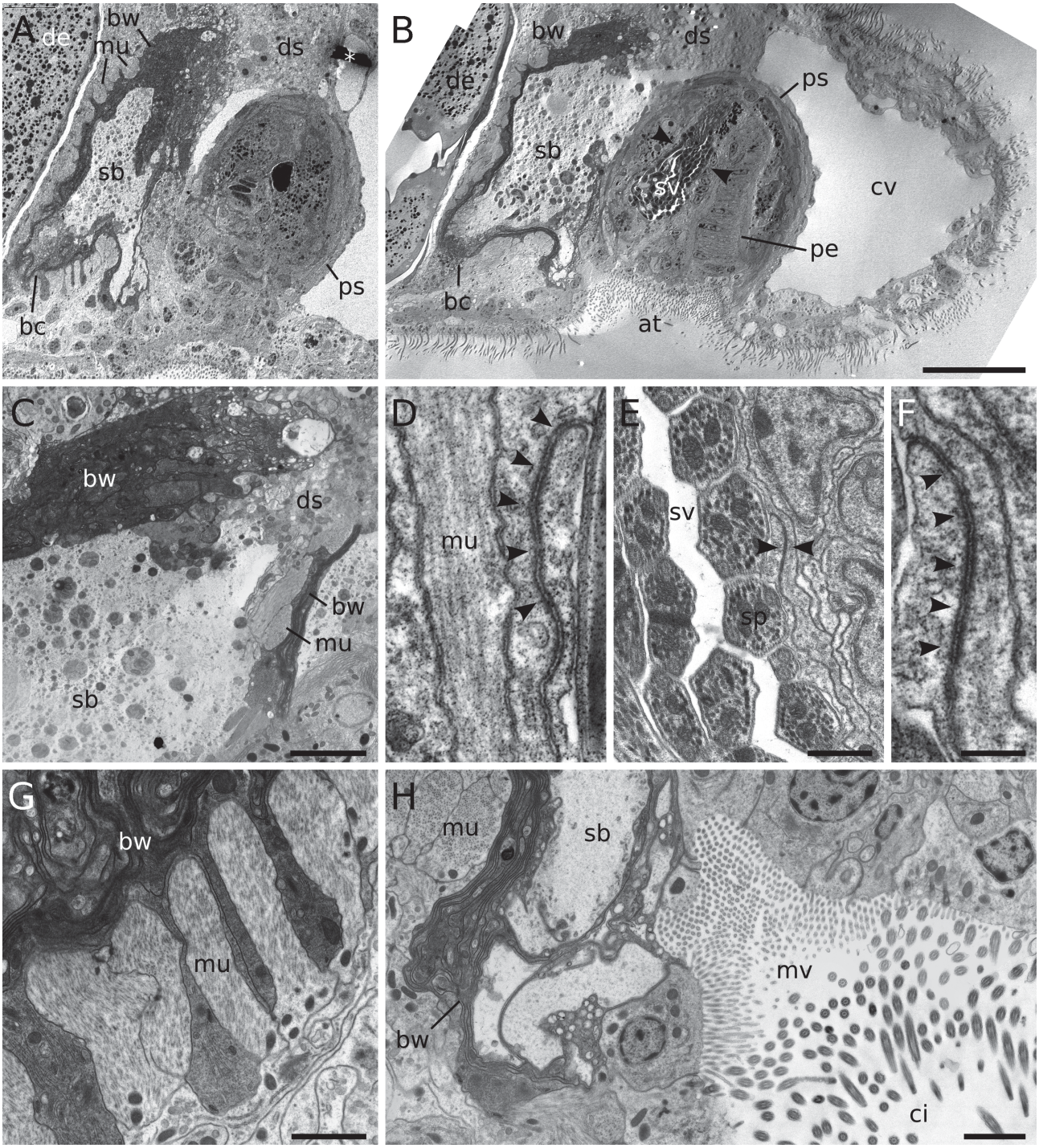

FIGURE 4. Electron micrographs (sagittal sections) of a mature animal, anterior to the left, dorsal up. (A,G) Sections slightly left of the median. (A,B) Overview of genital organs. (A) Anterior and ventral bursa wall *bw* lined with musculature *mu*. Note two ventral bursa coves—the anterior cove being the bursa cap *bc*—and the massive dorsal *bw*. Asterisk demarcates a speck of dust. (B–F, H) Sections very close to the median. (B) Penis *pe* and seminal vesicle *sv* in penis sheath *ps*. Arrowheads pointing at locations of septate junctions in *sv* epithelium magnified in (D–F). (C) Seminal bursa *sb* opens dorsally to the digestive syncytium *ds*. *mu* inside the posterior *bw*. (D–F) Septate junctions (arrowheads) of the epithelium lining the *sv*. (D) *mu* below the epithelium. (F) is a detail of (E). (G) Interspersed *mu* and *bw* between two ventral bursa coves. (H) Vaginal opening is lined with prominent microvilli *mv*, cilia *ci* from neighbouring epidermal cells. *at* atrium, *cv* chordoid vacuole, *de* developing egg, *sp* sperm. Scale bars: 20 µm in (B), 5 µm in (C), 2 µm in (G–H), 1 µm in (E), 0.25 µm in (F). Same scale in (A)+(B) and in (D)+(F).

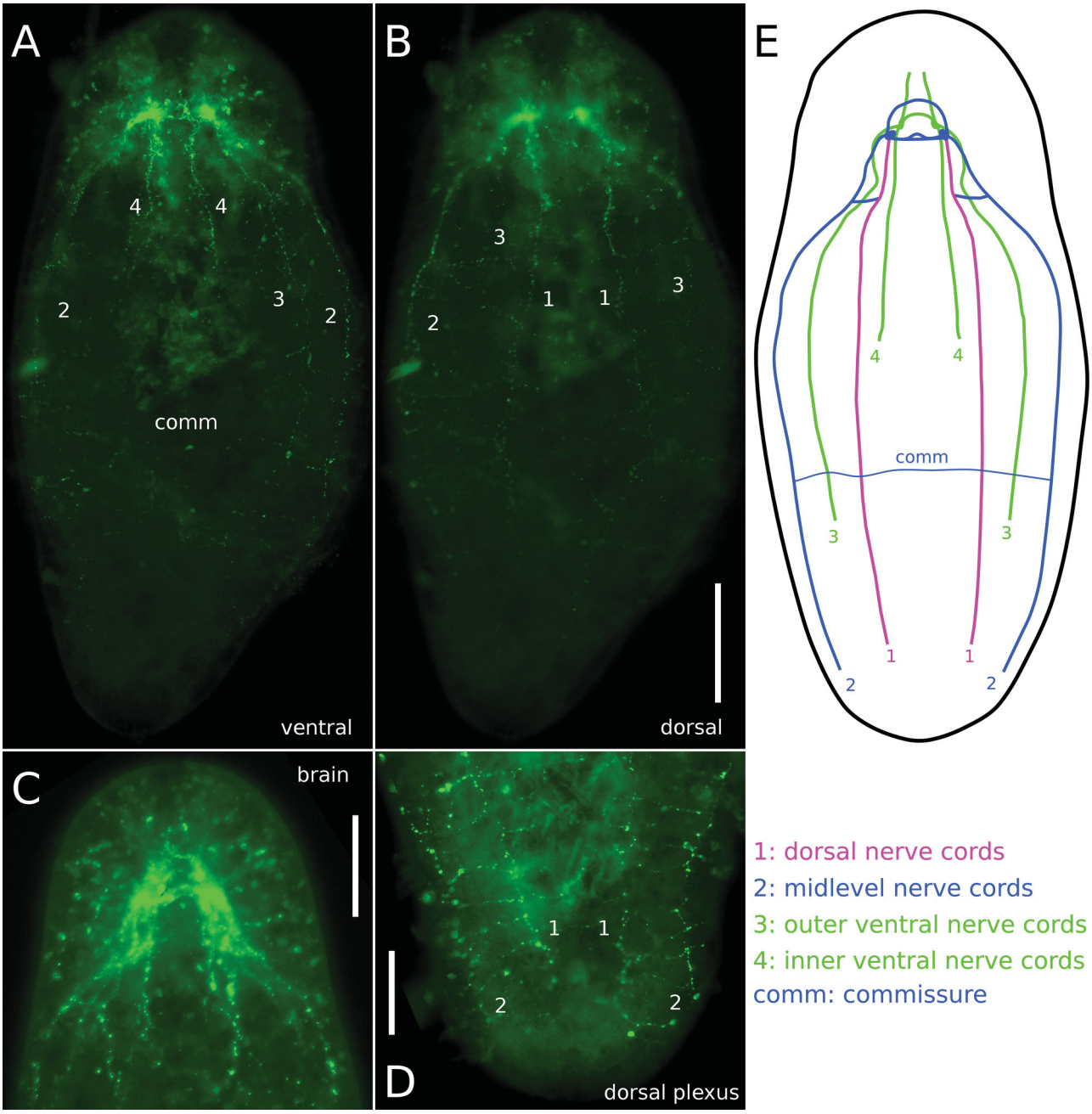

FIGURE 5. Serotonergic nervous system stainings of mature animals, anterior to the top. Ventral (A) and dorsal (B) views. (C) Confocal projection of anterior view with brain. (D) Dorsal nervous plexus of the posterior end. (E) Schematic drawing of the brain, the main longitudinal cords and the midlevel commissure. Scale bars: 100 µm in (A–B), 50 µm in (C–D).

Serotonergic nervous system. The main parts of the nervous system are arranged in longitudinal cords that are part of a peripheral plexus and that converge into an anterior (and interior) brain (Fig. 5). For the four pairs of main nerve cords, we follow the numbering system used by Achatz & Martinez (2012) for another isodiametrid species. Only the inner ventral nerve cords are closer together than the dorsal cords, followed by the outer ventral cords and, close to the body margin, the midlevel cords (Fig. 5ABE). The inner ventral cords are the shortest, not even reaching back to the level of the mouth, but also extending farther anterior than the other main cords. The outer ventral cords are second-shortest, going for about two thirds of the body length, while the dorsal and midlevel cords almost reach the posterior tip. The inner and outer ventral cords merge in the brain (serotonin-rich varicosity) and form a loop, while the most complex brain parts are built by the midlevel nerve cords, growing into a fully circular brain loop (Fig. 5CE). Large labeled cell bodies are found at the connection of dorsal cords and midlevel brain cords, and also a peculiar transversal connection between the longitudinal parts of the midlevel and the dorsal

cords could be observed (Fig. 5AE). Transversal commissures can be seen most prominently posterior of the mouth on the ventral/midlevel side (Fig. 5AE) and both anterior and posterior of the mouth on the dorsal side (Fig. 5D).

Cultures and embryonic development. *A. pisae* has been continuously cultured from May 2005 to January 2011, at which point the cultures were lost. The species was resampled at the same location in May 2011 and was kept in culture until July 2014, when the cultures perished again.

Thanks to our long-term cultures, we were able to observe the duration of the embryonic development until hatching (see Fig. 1E). Taking into account the intervals between observation time points at which new eggs and the hatched juvenile were observed, the shared interval between egg laying and hatching was 46–46.5 hours for all eggs (n=14). For two eggs, the observation intervals were particularly short, and they developed for at least 46 and maximally for 51.5 hours before hatching was noted. Thus, the embryonic development of *A. pisae* takes about (and usually less than) 2 days at 20 °C.

Discussion

Systematic classification. *Aphanostoma pisae* **sp. nov.** is recognised to be a member of the family Isodiametridae Hooge & Tyler, 2005 based on the presence of a muscular cylindrical (isodiametric) penis (with non-anastomising longitudinal muscle fibers) that is invaginated into a muscular penis sheath (Figs. 2–4) (Hooge & Tyler, 2005). Within the Isodiametridae, 22 genera have been described (Tyler *et al.*, 2006–2013). 19 of these genera are listed and scored with 22 characters in Table 4 of Hooge & Tyler (2005), useful as a key to genus identification. Another 2 genera were found to be isodiametrids by Hooge & Rocha (2006) and a new isodiametrid genus was described by Nilsson *et al.* (2011)[1]. Of these 22 genera, 10 have bursal appendages as seen in *A. pisae*. In *Diatomovora* Kozloff, 1965, *Isodiametra*, *Otocelis* Diesing, 1862 and *Raphidophallus* Kozloff, 1965, there is a characteristic sclerotised bursa nozzle present, while in *Aphanostoma*, *Haplocelis* Dörjes, 1968, *Postaphanostoma* Dörjes, 1968, *Praeconvoluta* Dörjes, 1968, *Proaphanostoma* (Dörjes, 1972) and *Proconvoluta* Dörjes, 1968 the bursal appendage may be formed as a non-sclerotised, cellular or muscular appendage. *Postaphanostoma* is different from our new species by the absence of a seminal vesicle, and *Proaphanostoma* and *Proconvoluta* by a penis that appears to be simply an inpocketing of the epidermis. *Haplocelis* is diagnosed with a long winding (non-sclerotised) bursa nozzle and it has a common genital pore that is directed posteriorly and a vagina that extends posterior to the male copulatory organ, unlike in our new species. The only two genera remaining, *Aphanostoma* and *Praeconvoluta*, are indistinguishable by consulting Table 4 given in Hooge & Tyler (2005).

The current diagnoses for the genera *Aphanostoma* and *Praeconvoluta* given by Dörjes (1968) and Faubel (1974) differ mostly in the presence of rhabdoids, a true seminal vesicle and a bursal appendage (*Aphanostoma*) or their absence (*Praeconvoluta*). Possessing rhabdoids, a seminal vesicle and a bursal appendage, the new species presented here is a member of the genus *Aphanostoma*.

In Table 4 of Hooge & Tyler (2005), the character "false seminal vesicle" is scored "absent" for both *Aphanostoma* and *Praeconvoluta*, which does not agree with Dörjes' genus diagnosis of *Praeconvoluta* and must thus be regarded as a mistake in that table. In addition, we observe that even for the description of the first species of the genus *Praeconvoluta*, *P. karinae*, Dörjes (1968) labeled a structure as "seminal vesicle" (his Figures 53B and 54D). Also Faubel (1974; 1977), Faubel & Regier (1983) and Hooge & Tyler (1999; 2003; 2008) described and labeled a seminal vesicle for the remaining seven *Praeconvoluta* species, but did not mention a false seminal vesicle (except Hooge & Tyler, 1999 for *P. tornuva*). Faubel (1977), Faubel & Regier (1983) and Hooge & Tyler (1999; 2003; 2008) also mentioned the existence of rhabdoids in their *Praeconvoluta* species, which is another difference between the genera *Aphanostoma* and *Praeconvoluta*. For *Praeconvoluta minor*, Faubel (1974) mentioned the absence of rhabdoids, and also changed the *Praeconvoluta* genus diagnosis to "Proximal seminal vesicle in penis sheath." (translated from German).

Based on these findings, we propose to update the genus diagnosis for *Praeconvoluta* as follows (see also Table 1)*: "*Uncoloured. Brain insunk. Frontal organ present. Mouth opening ventral. Rhabdoids present or missing. Ovaries and testes paired. Bursa seminalis simple, often with small cap. No bursa mouth piece. One or two genital

1. *Pharyngia* Nilsson, Wallberg & Jondelius 2011 seems like an odd candidate member of the isodiametrids: its penis ("penis papilla") is conical—and not isodiametrical!

openings, ventral. Penis strong, gland-rich, inverted into the muscular penis sheath. Proximal part of penis formed as granular vesicle. Proximal seminal vesicle in penis sheath. With or without false seminal vesicles. Penis sheath ellipsoid. Often strongly vacuolated."

The updated genus diagnosis for *Aphanostoma* is: "Coloured by characteristic bubbles, or uncoloured. Brain insunk. Frontal organ present. Mouth opening ventral. Rhabdoids in weak rows, or dispersed. Ovaries and testes paired. Two genital openings, very close to each other, or a single common opening. Bursa seminalis with muscular/cellular appendage, no sclerotised bursa nozzle. Sometimes concentrated actin spots in proximal bursa wall. Penis tube-like, inverted into the seminal vesicle, contained in a spherical penis sheath. Ciliated atrium present."

The difficulty to distinguish morphologically between *Aphanostoma* and *Praeconvoluta* is also reflected in the available molecular phylogenies, where representatives of both genera are found to be intermingling (Jondelius *et al.*, 2011; Nilsson *et al.*, 2011).

The Turbellarian Database (Tyler *et al.*, 2006–2013) currently recognises eight out of 35 listed *Aphanostoma* species as valid species names. These eight species are *A. album* Dörjes, 1968, *A. bruscai* Hooge & Tyler, 2003, *A. cavernosum* Meixner, 1938, *A. collinae* Hooge & Tyler, 2008, *A. elegans* Jensen, 1878, *A. rhomboides* Jensen, 1878, *A. sanguineum* Beklemishev, 1915 and *A. virescens* Ørsted, 1845. We found the species descriptions of *A. cavernosum* and *A. elegans* to be unrecognisable, while *A. sanguineum* was transferred to the genus *Pseudactinoposthia* by Hooge & Tyler (2005). Including *A. pisae*, this leaves the following six recognised *Aphanostoma* species (see also Table 1):

Aphanostoma album Dörjes, 1968
Aphanostoma bruscai Hooge & Tyler, 2003
Aphanostoma collinae Hooge & Tyler, 2008
Aphanostoma pisae **sp. nov.**
Aphanostoma rhomboides Jensen, 1878
Aphanostoma virescens Ørsted, 1845

Comparison of *Aphanostoma pisae* with other *Aphanostoma* species. Even including the *Praeconvoluta* species, *Aphanostoma pisae* is the first detailled species description of *Aphanostoma* for the Mediterranean (Table 1). *Aphanostoma chromocephalum* (incertae sedis) and *Aphanostoma rhomboides* were reported from the Adriatic Sea (Steinböck, 1933), but both identifications were considered only preliminary by the author himself, and no newer studies have confirmed these sightings. *A. pisae* is clearly not identical with *A. chromocephalum*, as the latter is described with a pigmented anterior end (Steinböck, 1933). Unlike *A. rhomboides*, *A. pisae* lacks pigmentation and the characteristic rhomboid arrangement of rhabdoids (Fig. 1C); also *A. pisae* is less than half the size of *A. rhomboides*. Some details concerning the genital organs still remain to be elucidated in *A. rhomboides*, such as whether it has an antrum or atrium, a bursal cap or a vaginal sphincter (Table 1).

A. pisae is among the smallest *Aphanostoma* species, like *A. collinae* being less than 1 mm in length. *A. pisae* also bears the shortest penis, and like *A. rhomboides* has a small ratio between penis length and body length. The orientation of the penis is usually ventrad. Most species of *Aphanostoma* have a common gonopore, but *A. pisae* and *A. rhomboides* have minimally separated male and female gonopores.

An overview of the distribution, body size, and the morphology of the genital organs for all 6 *Aphanostoma* and 8 *Praeconvoluta* species is provided in Table 1. It is apparent that the chief differences between the two genera are the shape of the penis sheath and the absence or presence of the bursal appendage. However, there is some nomenclatural confusion regarding the bursal appendage and bursal cap. Petrov *et al.* (2006) provided a glossary for some terms used for describing genital organs in acoels. They clarified that bursal mouthpieces are synonymous with bursal nozzles, a bursal appendage is “any cap-like or nozzle-like appendage of the bursa”, and a bursal cap is “an extension of the wall of the bursa composed of large cells or glandular elements”. This is corroborated by Hooge & Tyler (2008), page 19, where they wrote "the bursal appendage is in the form of a bursal cap that includes small actin-sclerotized bodies". In earlier descriptions of *Aphanostoma* and *Praeconvoluta* actin-sclerotised bodies may not have been recognised due to the unavailability of phalloidin stainings, but at least the definition by Petrov *et al.* (2006) suggests that a bursal cap is at the same time a bursal appendage. That is problematic insofar, as the genus diagnosis for *Praeconvoluta* specifies a "simple" bursa, as opposed to a bursa with bursal appendage (as in

TABLE 1. Comparison of sampling localities, body size and genital organs between members of the genera *Aphanostoma* and *Praeconvoluta*. 1 body length [µm]; 2 penis orientation: a anteriad, p posteriad, v ventrad, va ventro-anteriad, vp ventro-posteriad; 3 penis size [µm]; 4 normalised penis size (penis size/body length); 5 penis sheath: e ellipsoid, s spherical; 6 bursal appendage: + present, - absent, ? unclear; 7 bursal cap: + present, m musculous, c with concentrated actin spots, - absent, ? unclear; 8 genital openings; 9 vaginal sphincter: - absent, ? unclear; 10 antrum: c common antrum (that could also be called atrium), m male antrum (ciliated), - absent, ? unclear; 11 atrium: + present, - absent, ? unclear; 12 seminal vesicle with border other than the penis sheath: + labelled, d not labelled, but drawn, n not labelled.

Aphanostoma	author(s)	sampling localities	1	2	3	4	5	6	7	8	9	10	11	12
album	Dörjes, 1968	Sylt, Helgoland, Wilhelmshaven	800–1200	va	150	0.15	s	+	m	1	2	m	+	n
bruscai	Hooge & Tyler, 2003	Maine	600–800	v	60	0.09	s	+	c	1	1	-	+	n
collinae	Hooge & Tyler, 2008	Panama	480	va	53	0.11	s	+	c	1	-	c		n
pisae	this work	Marina di Pisa, Mediterranean	400–700	v	35	0.05	s	+	m	2	1	-	+	+
rhomboides	Jensen, 1878	Faroes, Iceland, Norway, Greenland, Barents Sea, Helgoland, Plymouth	1000–1500	vp	50	0.04	s	?	?	2	?	?	?	+
virescens	Ørsted, 1845	Faroes, Iceland, Norway, Greenland, White Sea	680	v	65	0.10	s	+	c	1	1	-	+	n
Praeconvoluta														
bocasensis	Hooge & Tyler, 2008	Panama	450–550	a	100	0.20	e	-	c	1	-	-	-	n
castinea	Hooge & Tyler, 2003	Maine	450–600	v	58	0.11	e	-	+	1	-	-	+	n
karinae	Dörjes, 1968	Helgoland	3000–4000	a	530	0.15	e	-	+	1	-	-	+	+
minor	Faubel, 1974	Sylt	1000–1300	a	135	0.12	e	-	+	1	1	-	+	+
schmidti	Faubel, 1977	Norway	450	p	45	0.10	e	-	+	2	-	-	+	+
stephania	Faubel & Regier, 1983	Northern North Sea	600	vp	24	0.04	e	-	+	2	-	-	-	+
tigrina	Hooge & Tyler, 2003	Maine	1200	a	120	0.10	e	-	-	1	-	-	+	n
tornuva	Hooge & Tyler, 1999	Maine	900	a	80	0.09	e	-	+	1	-	c		d

Aphanostoma). However, all *Praeconvoluta* species but one are described as having a bursal cap (Table 1), and thus a bursal appendage sensu Petrov *et al.* (2006). In *A. pisae* the anterior ventral cove of the bursa can be considered a bursa cap, and it appears to be non-sclerotised (Fig. 4AB).

In most descriptions of *Aphanostoma* and *Praeconvoluta* species, a distinct layer around the seminal vesicle different from the penis sheath is either not drawn or not labelled, or the penis sheath is labelled as "seminal vesicle" (e.g. *P. castinea* in Hooge & Tyler, 2003) (see also Table 1). In *A. pisae*, it is quite evident that there is a separate muscular and epithelial layer surrounding the seminal vesicle, that is different from the penis sheath (Figs. 2, 3C, 4D–F).

Anatomy. In *A. pisae*, we have identified an epithelial seminal vesicle, which is surrounded by musculature (Figs. 3C, 4D–F). Among previously described *Aphanostoma* species, only *A. rhomboides* has a labeled seminal vesicle, while a majority of *Praeconvoluta* species has a clearly drawn and labeled seminal vesicle (see Table 1). We document for the first time for these genera with electron microscopy, that the seminal vesicle is lined by a true epithelium (Fig. 4D–F). In most other *Aphanostoma* species, the seminal vesicle is not clearly lined and takes up most of the space in the penis sheath (e.g. Hooge & Tyler, 2008). Interestingly, we have not identified an adherens junction, but only a septate junction in the epithelium of the seminal vesicle.

The bursa wall cells are not made of an epithelium, at least we have not found septate or adherens junctions connecting these cells. The musculature associated with the bursa wall is usually situated at the outer side of the bursa wall (Fig. 4G), but at the dorsoposterior bursa wall, where the bursa opens into the digestive syncytium, we have found the musculature to be within the bursa wall (Fig. 4C). A bursa opening into the digestive parenchyma has been described for another isodiametrid, *I. pulchra* (Smith & Bush, 1991).

Musculature. In *A. pisae*, the body wall musculature (Fig. 3) is generally similar to that of other isodiametrids (Tyler & Rieger, 1999; Ladurner & Rieger, 2000; Hooge, 2001). Unlike *Isodiametra pulchra*, there are no ventral diagonal fibers that terminate at the midline before crossing over in *A. pisae* (Tyler & Rieger, 1999; Ladurner & Rieger, 2000). Hooge (2001) documents body wall musculature of other members of the Isodiametridae, specifically *Praeaphanostoma wadsworthi* Hooge & Tyler, 2003, *Otocelis sandara* Hooge & Tyler, 2003 and *Pseudaphanostoma smithrii* Hooge & Tyler, 2003; different from all aforementioned species, *A. pisae*'s longitudinal ventral muscle fibers from mouth to posteriad stop short at the anterior part of the bursa, instead of reaching the posterior end. In this respect and also in the presence of thick wedge-shaped parenchymal muscles at the anterior part of the animal, *A. pisae* is most similar to *Praeconvoluta tornuva* (Hooge & Tyler, 1999). Regrettably, there are no other depictions of the musculature of species of *Aphanostoma* or *Praeconvoluta* available, but even with the data at hand it seems that the pattern of muscle fibers not only of the genital organs, but also of the remaining body is useful for comparison and classification of acoel species (Hooge, 2001; Hooge & Tyler, 2005).

Serotonergic nervous system. To our knowledge, so far two studies address the serotonergic nervous system in isodiametrid acoels, in *Faerlea glomerata* Westblad, 1945 (Reuter *et al.*, 2001) and in *Isodiametra pulchra* (Achatz and Martinez, 2012). In the anterior part of *F. glomerata*, there are more transversal commissures than in *I. pulchra* and *A. pisae*, and the latter two share a very similar arrangement of nerves: four longitudinal nerve cords merging in the brain region, whereby the inner ventral cords are closer to the midline than the dorsal cords (Fig. 5).

As the staining of the serotonergic nervous system gets substantially weaker towards the posterior end of the animal, we cannot rule out the possibilty that the midlevel cords form a posterior loop. In *I. pulchra*, the main (lateral) nerve cords converge, but do not fuse posteriorly, as depicted in Achatz and Martinez (2012).

Cultures and embryonic developmental. *A. pisae* has been cultured for 5 and 3 years consecutively since 2005 and is, similar to *I. pulchra*, amenable to histological and molecular techniques, providing potential for using two closely related acoel species for comparative studies. The embryonic development of *A. pisae* has not been studied in detail yet, but the duration of about 2 days until hatching at 20 °C is also similar to *I. pulchra* (Ladurner & Rieger, 2000).

Conclusions and outlook

With this description of a new species of the genus *Aphanostoma*, we underscore the need for further study including sequencing of the type species of *Aphanostoma*, *A. virescens.* The 18S rDNA sequence attributed to *A. virescens* on GenBank is more similar to that of many species of *Isodiametra*, and less like that of the other

Aphanostoma species (see Jondelius *et al.*, 2011; Nilsson *et al.*, 2011). Second, there is hardly any difference in the generic diagnoses of *Aphanostoma* and *Praeconvoluta*, other than an elliptical penis sheath in *Praeconvoluta*, as opposed to a spherical penis sheath in *Aphanostoma.* Molecular analyses so far suggest both genera should be merged (see Jondelius *et al.*, 2011; Nilsson *et al.*, 2011). To help with this decision, it seems to be worthwhile to make an effort constructing a phylogenetic tree of the Isodiametridae with as many species as possible.

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